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Anaerobic Bioremediation of Benzene under Sulfate-Reducing Conditions in a Petroleum-Contaminated Aquifer

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The addition of sulfate to an anaerobic petroleum-contaminated aquifer in which benzene was a major soluble contaminant resulted in removal of benzene from the groundwater. The loss in benzene was associated with a decrease in sulfate along the groundwater flow path, relative to a conservative bromide tracer. Studies with [2-^{14}C]-acetate and molybdate demonstrated that sulfate reduction was the predominant terminal electron-accepting process (TEAP) in the sulfate-amended sediments, and studies with [^{14}C]benzene indicated that benzene oxidation was dependent upon sulfate reduction. Abundant ferrous iron in the subsurface likely prevented the generation of free sulfide in the groundwater during the field trial. Comparisons of benzene and sulfate depletion in the treatment zone indicated that benzene degradation could account for 53% of the sulfate depletion. These results suggest that the addition of sulfate stimulated the activity of benzene-degrading, sulfate-reducing microorganisms. This is the first field study demonstrating that it is possible to stimulate anaerobic benzene degradation in a petroleum-contaminated aquifer.

Introduction

Petroleum contamination of shallow aquifers frequently results in a rapid depletion of dissolved oxygen from the groundwater (*1*–*3*). Sites that have been extensively contaminated for long periods of time may have large areas of highly reduced sediment dominated by anaerobic processes (*4*–*10*). Remediation techniques based on the injection of oxygen into these anaerobic areas can be problematic due to the rapid consumption of oxygen by reduced products of anaerobic metabolism (*11, 12*). Fe(II) is especially problematic because oxidation of Fe(II) to insoluble iron(III) oxides may plug injection wells and/or the aquifer.

Problems with aerobic treatment of petroleum-contaminated aquifers has led to the consideration of anaerobic treatment processes (*13*–*18*). The addition of nitrate or sulfate as alternative electron acceptors has been found to accelerate the anaerobic degradation of some aromatic hydrocarbon contaminants in aquifers (*13, 14, 18*). However, none of these previous treatments were able to stimulate the degradation of benzene. This has diminished the potential for anaerobic remediation of these sites, because benzene is the aromatic hydrocarbon contaminant of greatest concern.

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Recent studies in our laboratory suggested that anaerobic benzene degradation could be stimulated with the addition of sulfate to aquifer sediments from a petroleum-contaminated aquifer located in Ponca City, OK (*16*). The predominant TEAP in these sediments was methane production, and benzene was degraded under methanogenic conditions (*9, 16*). However, when sulfate was added, the rate of benzene degradation was greatly stimulated. This was true in static bottle incubations as well as in a flow-through column system. Anaerobic benzene degradation in these sediments could also be stimulated with the addition of Fe(III) along with an electron-shuttle compound (*16*). However, the addition of sulfate was considered to be a better bioremediation option than the addition of Fe(III) because of the high electron-accepting capacity of sulfate and the ease of adding sulfate to the groundwater. Accumulation of free sulfide in situ was considered unlikely due to the presence of abundant ferrous iron in the sediment and a demonstrated potential for FeS precipitation during laboratory experiments (*9, 16*). Nitrate additions were not a viable bioremediation option because nitrate completely inhibited benzene degradation.

To evaluate whether the stimulation of anaerobic benzene degradation that was observed with the addition of sulfate in laboratory incubations would take place in situ, a pilot field study was initiated. The results of that study, reported here, demonstrate that the addition of sulfate greatly stimulated benzene degradation in situ. To our knowledge, this is the first example of the addition of sulfate promoting in situ remediation of benzene contamination.

Materials and Methods

Site Description and Installation. The bioremediation test site was located in Ponca City, OK, near the grounds of a major petroleum refinery. The long-term presence (>50 years) of contaminant hydrocarbons has resulted in an aquifer composed of highly reduced, methanogenic sediment. While free product is present in upgradient areas near the source of contamination, the treatment area for the pilot project was positioned at a downgradient location where the hydrocarbon contaminants are largely dissolved in the groundwater.

The test site is bounded at the downgradient end by a ravine into which the aquifer discharges (Figure 1). Within the treatment zone, the aquifer is approximately 0.9 m thick (3–4 m below surface) and is composed of coarse grain sediments of relatively high hydraulic conductivity (213 m/day). The hydraulic gradient calculated from water level data across the site (C98-184 through C98-189) averaged 0.003 and resulted in average seepage velocity of 2.1 m/day across the site. After 84 days of injection, the predicted length of the treatment zone is estimated at 176 m. Benzene is the major groundwater contaminant at this location and is present in the groundwater at concentrations up to 100 μM.

To facilitate the injection of sulfate into the aquifer, an injection gallery was installed perpendicular to the predominant groundwater flow direction and upgradient from a series of observation wells (Figure 1). Forty injection wells (25.4 mm diameter PVC) in two offset rows (20 per row) were installed in a rectangular shaped area allowing injection across a 30.5 m wide interval of the treatment zone. Slotted screens in all injection wells were placed at a depth of 2.7–3.7 m to ensure equal distribution across the contaminated thickness of the aquifer. Each injection well was capped and equipped with quick-connect fittings to allow the anaerobic transfer of injection solution and to prevent the introduction of air into the well.

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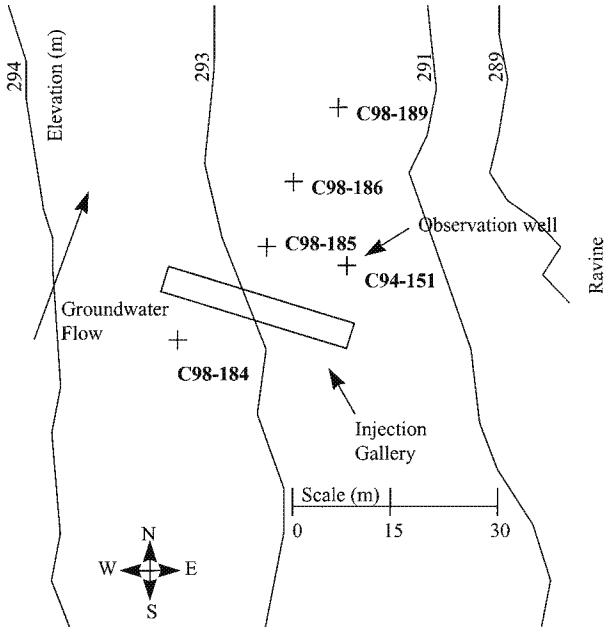

FIGURE 1. Plan view of the injection gallery and positions of the observation wells.

Observation wells were placed in a line perpendicular to and at varying downgradient distances from the injection gallery (Figure 1). Additional wells were positioned both upgradient from the injection gallery and at a downgradient location offset from the main line of wells (C94-151). All wells were screened over the same depth interval as the injection wells.

Preparation and Injection of Sulfate Solution. An anoxic sodium sulfate solution containing a bromide tracer was prepared daily and injected into the treatment zone via wells within the injection gallery. A 757-L (200 gal) solution of 8.0 mM sodium sulfate and 1.6 mM potassium bromide (Fisher Scientific Co., Springfield, NJ) was mixed in an enclosed stainless steel tank and sparged with nitrogen until the dissolved oxygen concentration decreased to 1 mg/L or less (YSI dissolved oxygen meter, Colorado Springs, CO). The entire tank remained under a nitrogen atmosphere during transport to the treatment area. Upon arrival at the injection gallery, the solution transfer line was gassed out with nitrogen prior to connection to the injection wells via the quick-connect fittings. Each injection well received approximately 19 L of the sulfate solution per day. During injection, nitrogen gas compensated for the volume displacement within the steel tank and prevented the introduction of air due to negative pressure buildup.

Groundwater Sampling and Analyses. Observation wells within the treatment zone were sampled weekly during the injection period and analyzed for a variety of organic and inorganic constituents. Benzene concentrations in HCl-preserved samples were measured with purge-and-trap gas chromatography (*19*). Briefly, groundwater samples were loaded into a discrete purging autosampler (OI-Analytical, DPM-16, College Station, TX) connected in series to a sample concentrator (OI-Analytical, series 4560, College Station TX) and a gas chromatograph (Hewlett-Packard, series 6890, Wilmington, DE). Benzene concentrations in samples were compared to standards (range 4−180 μM, detection limit 0.7 μM) prepared from methanol stock solutions.

Previous observations indicated that phenol might be an important intermediate during benzene degradation (*9*). Phenol concentrations in HCl-preserved samples were measured with high-pressure liquid chromatography (range 10−100 μM, detection limit 1.2 μM) and diode array detection (Hewlett-Packard, series 1100, Wilmington, DE) using an LC-18 column (Supelco, Bellefonte, PA) with an water:acetonitrile eluent (90:10).

Various inorganic constituents were also monitored during the pilot study. Sulfate and bromide concentrations (16−500 μM, detection limits 2.9 and 3.5 μM, respectively) in filtered (0.45-μm) samples were measured with ion chromatography (Dionex, series DX-100, Sunnyvale, CA). Sulfide concentrations in filtered (0.45-μm) samples were measured spectrophotometrically using *N,N*-dimethyl-*p*-phenylenediamine (Sigma Chemical Co., St. Louis, MO) (*20*) while Fe(II) concentrations in HNO_3-preserved samples were measured using the ferrozine assay (*21*).

Sediment Analyses. To analyze microbial processes during the study, sediments were collected with hand augers, dispensed into mason jars with no headspace, and shipped in coolers via overnight carrier to the laboratory as previously described (*9, 22, 23*). Upon arrival in the laboratory, all sediments were placed into an N_2-filled glovebag, homogenized, and dispensed into pressure tubes for use in experiments described below.

The TEAP in sediments was determined by monitoring the production of $^{14}CO_2$ and $^{14}CH_4$ from [2-^{14}C]acetate (specific activity 44.5 mCi/mmol, Sigma Radiochemicals, St. Louis, MO) as previously described (*22−24*). Briefly, 8−12 g of sediment was dispensed inside an N_2-filled glovebag into two sets of triplicate anaerobic pressure tubes. Upon removal from the glovebag, all tube headspaces were gassed out with N_2:CO_2 (95:5) that had been passed over hot copper filings to remove residual oxygen. One set of tubes received 1.5 mL of anaerobically prepared 1 mM Na_2MoO_4 to inhibit potential sulfate reduction. All tubes received approximately 0.25 μCi of [2-^{14}C]acetate, and the production of $^{14}CO_2$ and $^{14}CH_4$ was monitored using a gas proportional radiochromatography detector (IN/US Systems Inc., GC-Ram, Tampa, FL) connected to a gas chromatograph (Shimadzu, GC-8A, Kyoto, Japan).

In a similar manner, the benzene degradation potential of collected sediments was assessed using radiotracer methods as previously described (*22−25*). [U-^{14}C]Benzene (specific activity 58.2 mCi/mmol, Sigma Radiochemicals, St. Louis, MO) was added (0.6−0.8 μCi) to triplicate sets of anaerobic pressure tubes containing 8−12 g of sediment, and the production of $^{14}CO_2$ and $^{14}CH_4$ was monitored over time by gas chromatography and proportional counting.

Results and Discussion

Establishing Sulfate-Reducing Conditions with the Addition of Sulfate. With the exception of well C98-189, observation wells installed within the treatment zone were sampled on a weekly basis 10 weeks prior to the injection of sulfate in order to determine baseline concentrations of benzene, sulfate, bromide, and dissolved Fe(II). Well C98-189 was installed just prior to initiation of sulfate injection and was incorporated into the weekly groundwater sampling program thereafter. Baseline benzene concentrations ranged from 10 to 105 μM with an overall average of 55 μM before sulfate injection began (Figure 2). Baseline bromide concentrations ranged from 8 to 15 μM while sulfate concentrations ranged from 14 to 383 μM. Concentrations of dissolved Fe(II) were comparable at all sites and averaged 312 μM (data not shown).

Sulfate and bromide concentrations increased rapidly in downgradient observation wells once the injection period began (Figure 2). Elevated bromide concentrations signaled the arrival of the injection solution at the observation wells. Minor fluctuations in bromide concentrations during the treatment period likely resulted from the once daily input of the injection solution. There was no increase in bromide in the well upgradient from the injection zone.

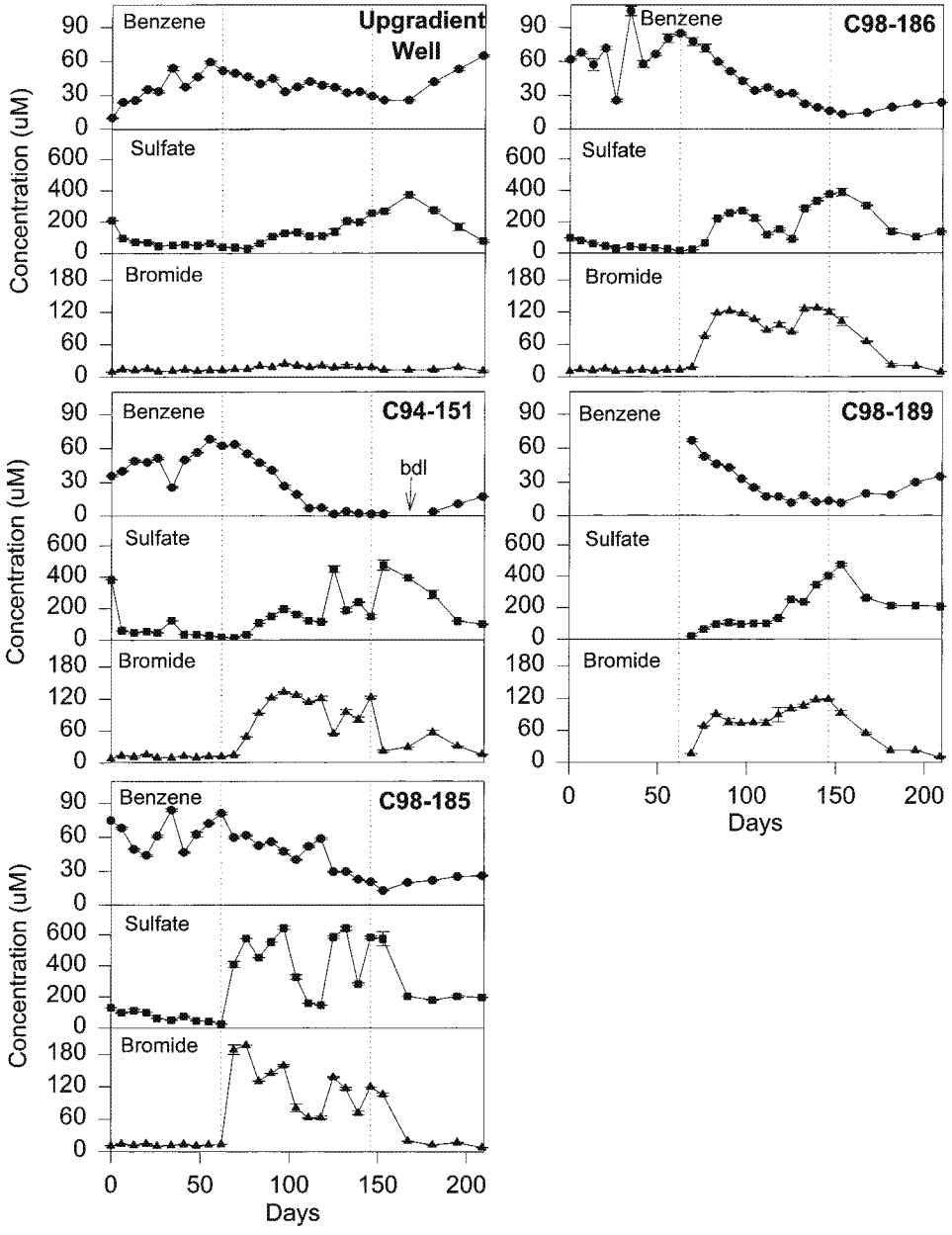

FIGURE 2. Groundwater concentrations of benzene, sulfate, and bromide in observation wells positioned both upgradient and downgradient (C94-151, C98-185, C98-186, and C98-189) of the injection gallery. Well C98-189 was installed just prior to initiation of sulfate injection. Results are averages of triplicate analyses. Some error bars are obscured by the plot symbol. Dashed lines indicate the beginning and the end of the injection period (bdl, below detection limit).

Increases in bromide in the downgradient wells were accompanied by an increase in sulfate (Figure 2). This indicated that the increase in sulfate was primarily the result of the sulfate injection. There was a much more gradual increase in the sulfate concentration at the upgradient site, which most likely originated from surface water recharge high in sulfate. After the injection period, bromide concentrations decreased to levels observed prior to the injection period, and sulfate concentrations slowly decreased, but monitoring was not continued long enough to observe a decrease to preinjection levels.

Although sulfate concentrations increased in the treatment zone during sulfate addition, there was also significant removal of sulfate from the groundwater as it moved through the treatment zone as evidenced by sulfate to bromide ratios in the groundwater that were significantly lower than the sulfate to bromide ratios in the injection solution (Figure 3). The sulfate to bromide ratios generally decreased with increasing distance along the groundwater flow path, indicating that sulfate continued to be removed as the groundwater moved out to well C98-189. The sulfate to bromide ratios at well C94-151 were generally as low or lower than those observed in C98-189, which suggested that this area, slightly off the main axis of the flow from the injection gallery, was a zone of intense sulfate consumption.

Sulfate removal from the groundwater was probably the result of sulfate reduction because analysis of the TEAP in the sediments demonstrated that sulfate reduction was the predominant TEAP (Figure 4). [2-^{14}C]Acetate was converted solely to $^{14}CO_2$ in sediments collected near the main line of observation wells positioned downgradient of the injection gallery (Figure 4). This contrasts with the conversion of [2-^{14}C]acetate primarily to $^{14}CH_4$ that was previously reported for these sediments (*9, 16*). In sediments, [2-^{14}C]acetate is converted primarily to $^{14}CH_4$ under methanogenic conditions and primarily to $^{14}CO_2$ under sulfate-reducing and Fe(III)-

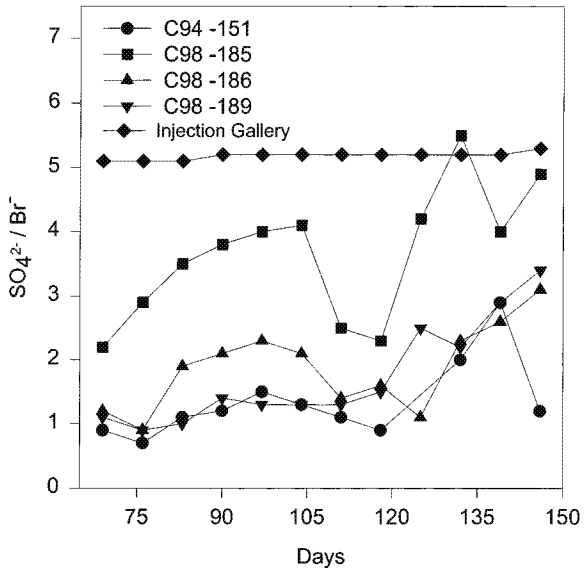

FIGURE 3. Sulfate to bromide ratios calculated for treatment zone wells at varying distances from the injection gallery. Wells C98-185, C98-186, and C98-189 are positioned at increasing downgradient distances in a perpendicular line from the injection gallery. Well C94-151 is positioned near the injection gallery but off the main line of observation wells.

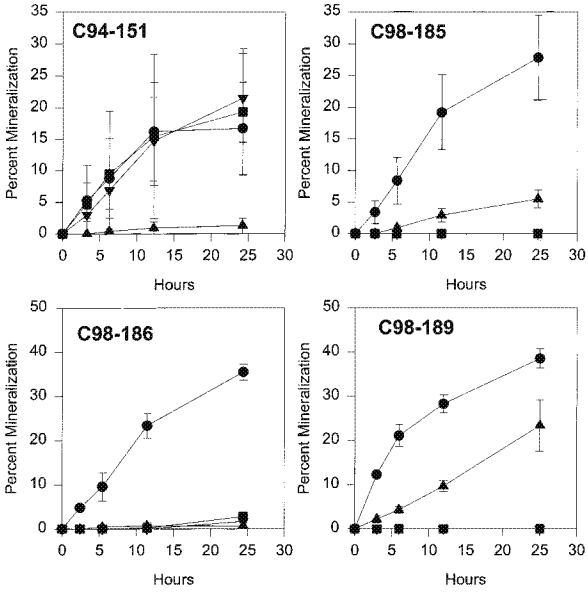

FIGURE 4. Mineralization of [2-^{14}C]acetate in treatment zone sediment samples collected at the end of the treatment period. Well C98-189 was installed just prior to the beginning of the injection period. Results are averages of triplicate analyses (●, $^{14}CO_2$ no additions; ▲, $^{14}CO_2$ molybdate added; ■, $^{14}CH_4$ no additions; ▼, $^{14}CH_4$ molybdate added).

reducing conditions. Sulfate reduction can be distinguished from Fe(III) reduction by comparing the rates of $^{14}CO_2$ production in the presence and in the absence of molybdate. The addition of molybdate, a specific inhibitor of sulfate reduction (*26*), inhibited the production of $^{14}CO_2$ relative to sediments not containing molybdate (Figure 4), suggesting that acetate oxidation was coupled to sulfate reduction and not Fe(III) reduction. Production of $^{14}CH_4$ and molybdate-inhibited $^{14}CO_2$ observed in sediments collected near C94-151 indicated a sulfate-limited TEAP at this location where neither methanogenic processes nor sulfate reduction were dominant. This result is consistent with the suggestion of extensive sulfate consumption near this location.

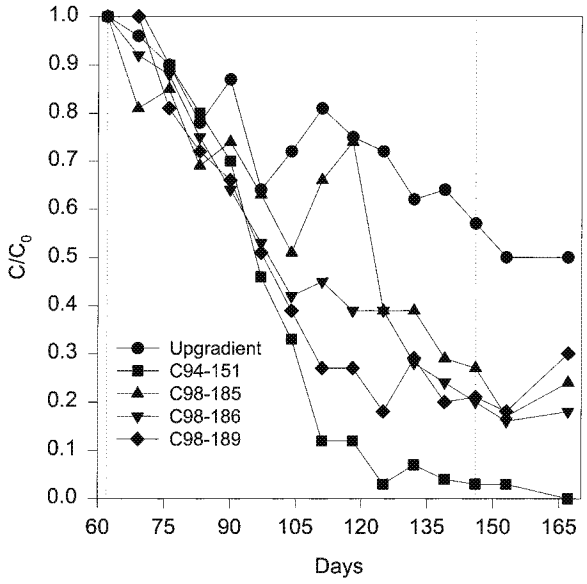

FIGURE 5. Relative decreases in benzene concentrations at the observation wells. All concentrations are normalized to observed benzene concentrations measured just prior to the start of the injection period. Dashed lines indicate the period of sulfate injection.

Free sulfide was not detected in groundwater samples at any time during the injection period. However, as noted above, the groundwater contained substantial amounts of dissolved Fe(II). It is likely that any sulfide produced would have precipitated as iron sulfides. In previous column studies conducted with sediments from this site (*16*), there was significant sulfate reduction but no accumulation of dissolved sulfide because the sulfide was precipitated out in the sediments which became progressively darker over time.

Stimulation of Benzene Degradation with the Addition of Sulfate. Sulfate consumption in the downgradient wells was associated with a significant decrease in the relative concentrations of benzene (Figure 5). In one well (C94-151), benzene concentrations dropped from 63 μM to below detection (detection limit 0.7 μM). Large decreases in benzene concentrations were also observed in wells C98-185 (from 82 to 22 μM), C98-186 (from 86 to 17 μM), and C98-189 (from 68 to 14 μM) during the treatment period. The decreases in benzene concentrations in the treatment zone were not the result of dilution with the injection water. Bromide concentrations in the downgradient observation wells averaged 5% (± 2%) of the bromide in the injection solution, and the cumulative volume of injection solution could only account for a maximum benzene loss of 4% due to dilution (total volume of injection solution divided by total liquid volume of the treatment zone). This contrasts with an overall removal average of 83 ± 10% of initial benzene concentrations from groundwater in wells downgradient from the injection gallery (Figure 5) and complete removal of benzene at site C94-151.

Benzene concentrations also decreased (43%) in the upgradient well (C98-184) during the injection period even though a lack of increase in bromide indicated that this zone was not influenced by the injection solution. This decrease in benzene may have resulted from the apparent increased inputs of sulfate from surface water recharge during this period. Benzene losses within the treatment zone were about twice as great as the loss of benzene observed at the upgradient site.

The loss of benzene coincident with sulfate removal from the groundwater suggested that benzene degradation was coupled to sulfate reduction within the treatment zone. To further investigate this possibility, sediments collected after the injection period were incubated anaerobically with

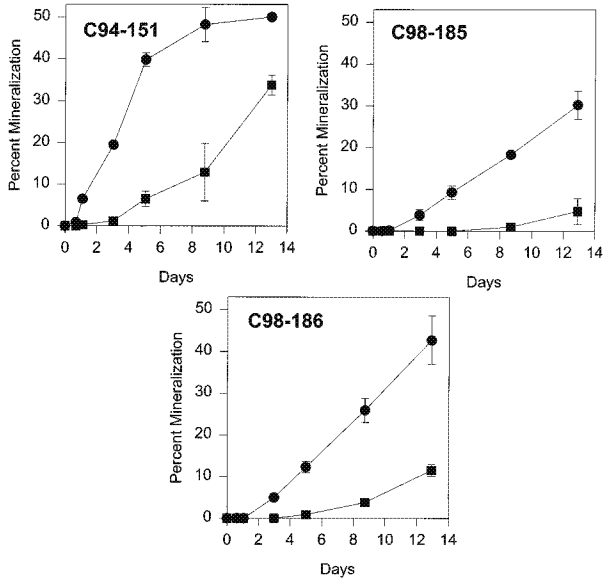

FIGURE 6. Mineralization of [U-^{14}C]benzene in sediments collected within the treatment zone at the end of the treatment period. Results are averages of triplicate analyses (●, $^{14}CO_2$ no additions; ■, $^{14}CO_2$ molybdate added).

[U-^{14}C]benzene. $^{14}CO_2$ was produced without a lag period, suggesting that organisms in the sediments were mineralizing benzene in situ (Figure 6). The addition of molybdate inhibited benzene mineralization, which indicated that benzene oxidation was coupled to sulfate reduction. Indirect evidence that benzene was being oxidized by sulfate reduction was the finding that no phenol could be detected in the groundwater. Previous investigations in our laboratory with sediment collected from this site indicated that phenol is a benzene degradation intermediate under methanogenic conditions but not when sulfate reduction is the TEAP (*9*, *16*). Further evidence for the stimulation of benzene oxidation coupled to sulfate reduction with the addition of sulfate to the treatment zone was the finding that the abundance of sulfite reductase genes in the aquifer sediments increased as much as 2 orders of magnitude following the addition of sulfate (*27*). This result is consistent with the hypothesis that sulfate injection stimulated an increase in the sulfate-reducing population of the treatment zone sediments.

The loss of benzene could account for a large percentage of the loss of sulfate during the injection period. If it is assumed that, as previously shown (*25*), benzene degradation was coupled to sulfate reduction according to

$$C_6H_6 + 3.75SO_4^{2-} \rightarrow 6CO_2 + 3.75S^{2-} + 3H_2O$$

then the calculated removal of benzene from the groundwater in the treatment zone would have been responsible for the removal of 53% of the sulfate that was consumed in the treatment zone (Table 1).

Implications for Anaerobic Bioremediation of Benzene. The results demonstrate that the addition of sulfate can stimulate anaerobic benzene degradation in this petroleum-contaminated aquifer. Although other attempts have been made to enhance anaerobic benzene degradation in situ with alternative electron acceptors (*13*, *14*, *18*), to our knowledge, this is the first successful field demonstration in which the anaerobic degradation of benzene has been accelerated in situ. The field trial was not optimized nor of sufficient duration to demonstrate complete removal of benzene in all wells. Nevertheless, anaerobic benzene degradation was stimulated in situ, demonstrating proof of concept.

TABLE 1. Mass Loss Calculations of Benzene and Sulfate Observed during the Injection Period at the Ponca City Site

well	benzene removed (mol)[a]	sulfate removed (mol)[b]	corrected benzene removed (mol)[c]	corrected sulfate removed (mol)[d]	sulfate consumed due to benzene oxidation (mol)[e]	% sulfate consumed due to benzene oxidation
C94-151	90.3	308	57.3	483	215	44.5
C98-185	88.5	121	55.5	296	208	70.2
C98-186	101	292	67.8	467	254	54.4
C98-189	78.3	227	45.3	402	170	42.2
averages	89.5	237	56.5	412	212	52.8

[a] Calculated from the observed decrease in benzene concentrations during the treatment period. [b] Calculated from predicted sulfate concentrations relative to bromide minus observed sulfate concentrations during treatment. [c] Calculated from observed benzene removals within treatment zone wells after subtraction of upgradient removal trend. [d] Calculated from observed sulfate concentrations within treatment zone wells subtracted from the sum of the predicted sulfate concentrations relative to the bromide tracer (corrected for upgradient Br^- concentration) and observed upgradient sulfate concentrations. [e] Theoretical molar ratio of sulfate to benzene = 3.75 if benzene mineralization is coupled to sulfate reduction according to $C_6H_6 + 3.75SO_4^{2-} \rightarrow 6CO_2 + 3.75S^{2-} + 3H_2O$. Corrected benzene removed multiplied by 3.75.

The addition of sulfate to the Ponca City aquifer resulted in a virtually immediate stimulation of benzene removal. This is in contrast to column studies with sediments from this aquifer in which there was a lag period before the addition of sulfate stimulated benzene degradation (*16*). The rapid loss of benzene in the field study is consistent with the results of laboratory incubations of sediments without flowing water in which the addition of sulfate immediately stimulated benzene degradation (*16*). The differences in these various investigations may reflect the amount of sediment disturbance in the different treatments. Construction of the columns required significantly more handling and processing of the sediments than did the laboratory incubations without flowing water. The in situ treatment was accompanied by no physical disruption of the microbial environment.

As previously discussed (*16*), the addition of sulfate as an electron acceptor to promote the removal of benzene has several potential advantages over aerobic bioremediation strategies, especially for heavily contaminated source zones. Sulfate does not react with reduced metabolites such as Fe(II) or sulfide. Thus, unlike oxygen, sulfate is not consumed by abiological processes unrelated to benzene degradation, and the addition of sulfate does not result in plugging of the aquifer with precipitated iron(III) oxides. Sulfate is much more soluble than oxygen, and thus higher concentrations can be added to groundwater. Furthermore, sulfate has twice the electron-accepting capacity of oxygen (*16*). Several inexpensive sources of sulfate are available. Although for this field trial sulfate was added in a solution, for larger-scale, longer-term applications it may be more economical to emplace solid sulfate sources in an upgradient location and permit groundwater flow to solubilize and distribute the sulfate.

However, the addition of sulfate may not be an appropriate remediation approach for all petroleum-contaminated aquifers. Previous studies have suggested that some petroleum-contaminated aquifers may not contain the appropriate benzene-degrading, sulfate reducers (*28*). In these instances, benzene continues to persist when sulfate is added unless the sediments are also inoculated with benzene-degrading, sulfate-reducing microorganisms (*28*). Thus, analysis of the microbial community and/or laboratory studies to assess the potential for benzene oxidation coupled to sulfate reduction should be considered prior to application of this bioremediation strategy.

Acknowledgments

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